[CANCER RESEARCH 57, 2839–2842, July 15, 1997]

Advances in Brief

Benzene Poisoning, a Risk Factor for Hematological Malignancy, Is Associated with the *NQO1* ^{609}C→T Mutation and Rapid Fractional Excretion of Chlorzoxazone[1]

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Abstract

Benzene is a ubiquitous occupational hematotoxin and leukemogen, but people vary in their response to this toxic agent. To evaluate the impact of interindividual variation in enzymes that activate (*i.e.*, CYP2E1) and detoxify (*i.e.*, NQO1) benzene and its metabolites, we carried out a case-control study in Shanghai, China, of occupational benzene poisoning (BP; *i.e.*, hematotoxicity), which we show is itself strongly associated with subsequent development of acute nonlymphocytic leukemia and the related myelodysplastic syndromes (relative risk, 70.6; 95% confidence interval, 11.4–439.3). *CYP2E1* and *NQO1* genotypes were determined by PCR-RFLP, and CYP2E1 enzymatic activity was estimated by the fractional excretion of chlorzoxazone ($fe_{6\text{-}OH}$) for 50 cases of BP and 50 controls. Subjects with both a rapid $fe_{6\text{-}OH}$ and two copies of the *NQO1* ^{609}C→T mutation had a 7.6-fold (95% confidence interval, 1.8–31.2) increased risk of BP compared to subjects with a slow $fe_{6\text{-}OH}$ who carried one or two wild-type *NQO1* alleles. In contrast, the CYP2E1 *PstI/RsaI* polymorphism did not influence BP risk. This is the first report that provides evidence of human susceptibility to benzene-related disease. Further evaluation of susceptibility for hematotoxicity and hematological malignancy among workers with a history of occupational exposure to benzene is warranted.

Introduction

Benzene causes hematotoxicity and ANLL[3] (1) and recently has been associated with increased risk of MDS and NHL (2). There are multiple clinical reports suggesting that people vary greatly in their susceptibility to adverse health outcomes from benzene exposure (3). One reason for this could be interindividual variation in metabolic activation and detoxification.

Benzene is metabolized by the hepatic enzyme CYP2E1 to benzene oxide (4), which spontaneously forms phenol and is itself further metabolized by CYP2E1 to hydroquinone (Ref. 5; Fig. 1). Hydroquinone and related hydroxy metabolites are converted in the bone marrow by myeloperoxidase to benzoquinones (6, 7), which are potent hematotoxic and genotoxic compounds that can be converted by NQO1 back to less toxic hydroxy metabolites (Ref. 8; Fig. 1). We therefore hypothesized that high CYP2E1 and low NQO1 activity would make people susceptible to BP (*i.e.*, hematotoxicity).

Here, we report the results of a retrospective cohort study of 11,177 benzene-exposed workers in Shanghai, China, that evaluates the relationship between BP and subsequent development of hematological malignancies and related disorders and of a case-control comparison between 50 workers with a history of BP and 50 unexposed controls for *CYP2E1* and *NQO1* genotypes and CYP2E1 enzymatic activity estimated by the fractional excretion of chlorzoxazone as the 6-hydroxy metabolite ($fe_{6\text{-}OH}$) (9).

Materials and Methods

Cohort Study of Hematological Malignancies and Related Disorders among Workers with BP

We have previously described in detail the methods used to carry out a retrospective cohort study of 74,828 benzene-exposed workers employed during the period of 1972–1987 in 12 cities in China (2). In this report, we focus on a subset of 11,177 workers who were employed in benzene-exposed jobs in Shanghai, China. In 1988, we initiated a retrospective ascertainment of all incident cases of hematological diseases (*i.e.*, leukemias, lymphomas, other hematolymphoproliferative malignancies, and related nonmalignant hematopoietic disorders, such as MDSs) and deaths from all causes occurring during the period of 1972–1987 using occupational personnel and health records and, if needed, contacts with next-of-kin, work colleagues, or physicians (2). Available medical records and pathological samples were reviewed by expert Chinese and United States hematopathologists (10). Historical benzene exposure during subjects' employment at the factories studied (1949–1987) was estimated by trained field personnel (11). Estimates of cumulative exposure to benzene (in ppm-years) were calculated by summing over the historical time-specific exposure estimates during the time interval of employment in the factory.

Benzene-exposed workers were periodically screened at their factories for evidence of BP. The published criteria used to diagnose BP are as follows: (*a*) the subject's total WBC count is <4,000/μl, or the WBC count is between 4,000 and 4,500/μl and platelet count is <80,000/μl, repeatedly demonstrated in peripheral blood counts performed over several months; (*b*) employment in a factory with documented benzene exposure for at least 6 months; and (*c*) exclusion of other causes of abnormal blood counts (12). BP is generally reversible following cessation of benzene exposure (3). Ascertainment of BP occurring at any time during employment in the factories studied was carried out through review of occupational personnel and health records.

Statistical Analysis. Poisson regression was used to estimate the relationship between documented history of BP and risk of total hematolymphoproliferative malignancies (ICD9 200–208) and MDS. MDS were combined with the malignant conditions because growing evidence suggests that these disorders are precursors to ANLL (13), and these disorders have generally not been distinguished from ANLL in previous epidemiological studies. History of BP was considered as a time-dependent exposure variable; person-time before diagnosis and all person-time for those who never had a diagnosis of BP were

Received 2/13/97; accepted 5/21/97.

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[1] This study was supported in part by National Institute of Environmental Health Sciences Grants P42ES04705 and P30ES01896. S. C. and K. B. M. are trainees of the University of California Toxic Substances Program.

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[3] The abbreviations used are: ANLL, acute nonlymphocytic leukemia; $fe_{6\text{-}OH}$, fractional excretion of chlorzoxazone; BP, benzene poisoning; MDS, myelodysplastic syndromes; NHL, non-Hodgkin's lymphoma; TWA, time-weighted average; OR, odds ratio; CI, confidence interval; NQO1, NADPH:quinone oxidoreductase; CYP2E1, P4502E1.

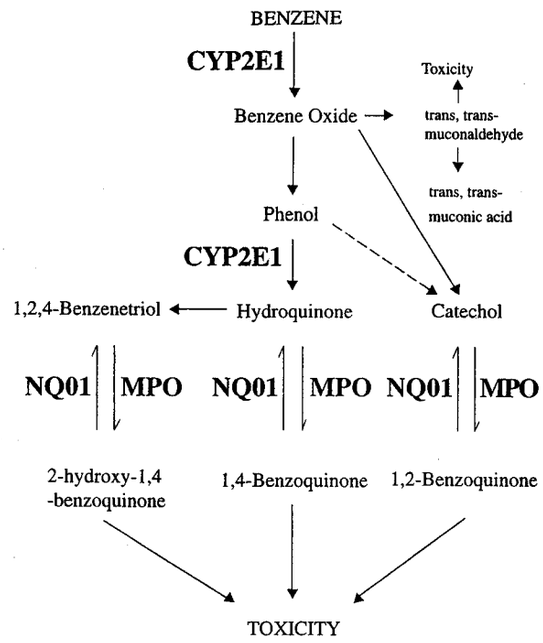

Fig. 1. The role of CYP2E1, NQO1, and MPO (myeloperoxidase) in benzene metabolism.

assigned to the non-BP group, whereas person-time after diagnosis of BP was assigned to the BP group. Cases were similarly assigned to the BP and non-BP groups. The relative risk estimate is the ratio of the empirical incidence rates of hematolymphoproliferative malignancies combined with MDS in benzene-poisoned workers and in workers with no history of BP, with adjustment for age (5-year intervals) and sex, and for cumulative benzene exposure (<10, 10–99, 100–399, and 400+ ppm-yr).

Case-Control Study of BP

Subject Enrollment. We initiated the case-control study of BP in 1992. Eligibility criteria for BP cases were as follows: (*a*) to have a confirmed diagnosis of BP in Shanghai after 1970; (*b*) to be alive at the time of the study, a resident of Shanghai, and to be ≤65 years of age as of 1992; (*c*) to have no prior history of cancer, therapeutic radiation, or chemotherapy; and (*d*) to not be pregnant at time of study. Also, we used more stringent diagnostic criteria (*i.e.*, WBC count ≤3500/μl) than the published criteria described above (12) to minimize the probability that subjects with low but normal WBC counts had been incorrectly diagnosed with BP. Of the 103 workers with BP identified from the Shanghai cohort, 77 were diagnosed after 1970, 75 were alive in 1992, and 54 satisfied the remaining eligibility criteria. Fifty of the 54 eligible cases (92%) agreed to participate in the study.

Fifty controls were selected from two workplaces in Shanghai without benzene exposure: one workplace manufactured sewing machines and the other was an administrative facility. Eligibility criteria for controls included no history of occupational exposure to benzene, other marrow-toxic chemicals, or ionizing radiation, in addition to criteria *c* and *d* described above. Controls were frequency matched by age (5-year intervals) and sex to cases. Ninety-four % of eligible controls selected for this study agreed to participate.

Current exposure to benzene was monitored by organic vapor passive dosimetry badges (No. 3500, 3M Co., St. Paul, MN). Badges were worn by each worker currently working in a factory with potential for benzene exposure for several days during the 1–2-week period prior to the clinical phase of the study and were analyzed by gas chromatography with flame ionization detection.

The protocol was explained to potential participants, and informed consent was obtained using Institutional Review Board-approved procedures. Subjects were asked to refrain from eating solid foods after dinner the night before and the morning of the clinical phase of the study. During the morning of the study, subjects voided, received 250 mg of chlorzoxazone, and collected all urine voided over the next 8 h. Samples were kept on ice until processing (*i.e.*, volume determination and aliquoting) was completed. Subjects were allowed to ingest only noncaffeinated soft drinks for the first 2.5 h, after which they were provided with a light lunch. On the same day, a 27-ml volume of blood was collected from each subject and fractionated into plasma, buffy coat, and RBCs. Each subject's height and weight were measured, and a questionnaire was administered by a trained interviewer that collected information on age, sex, current and lifelong tobacco use, alcohol consumption, and medical and occupational history.

Laboratory Analysis. Seventy-two to 80% of the hepatic metabolism of chlorzoxazone to its 6-hydroxy metabolite has been attributed to CYP2E1 activity (14, 15). In a pilot study conducted for the current investigation, De Vries *et al.* (9) demonstrated that the $fe_{6\text{-}OH}$ correlated strongly ($r = 0.89$) with a full pharmacokinetic evaluation of hepatic chlorzoxazone metabolism. In the current study, 6-hydroxychlorzoxazone in urine samples was measured by high-performance liquid chromatography following β-glucuronidase treatment as described (16). The $fe_{6\text{-}OH}$ was calculated by multiplying the 6-hydroxychlorzoxazone urine concentration by the total urine volume excreted over 8 h and then dividing by the 250-mg dose of chlorzoxazone.

A nested PCR assay (17) was used to measure a RFLP in the 5′-flanking region of the *CYP2E1* structural gene, identified by *Pst*I and *Rsa*I restriction sites in complete disequilibrium (18). The major (wild-type) allele is designated c1, and the minor allele is designated c2. A homozygous $^{609}C\rightarrow T$ mutation in *NQO1* is polymorphic in humans (19–21) and causes a complete loss of enzyme activity among homozygotes (19). The *NQO1* $^{609}C\rightarrow T$ mutation was measured by a PCR-RFLP assay (22).

Statistical Analysis. Unconditional logistic regression was used to estimate the relative risk of BP. Subjects were divided into those with a relatively rapid or slow $fe_{6\text{-}OH}$ based upon the median $fe_{6\text{-}OH}$ value in controls (*i.e.*, 51%) and into those who were homozygous for each genetic polymorphism *versus* individuals who carried one or two copies of the major alleles. ORs adjusted for the matching variables (age and sex) and for body mass index, current alcohol use (yes/no), and smoking status (yes/no) are reported. Two-tailed *P* values <0.05 were considered statistically significant.

Results

Cohort Study of Benzene Hematotoxicity and Risk of Subsequent Hematological Malignancies and Related Disorders. Among 11,177 benzene-exposed workers in Shanghai, 103 were diagnosed with BP. Three subjects with BP subsequently developed hematological malignancies or related disorders (*i.e.*, one each of ANLL, MDS, and NHL) within 4–14 years after diagnosis of BP, whereas 7 subjects among the 11,074 workers without an antecedent diagnosis of BP were diagnosed with these conditions (*i.e.*, three of NHL, two of ANLL, and one each of chronic myelogenous leukemia, multiple myeloma, and MDS; Table 1). BP was associated with a highly significant 42-fold increased risk for subsequent development of any hematological malignancy or MDS, and a 71-fold risk for the subgroup of ANLL or MDS (Table 1); the latter two disorders have been most consistently and strongly associated with benzene exposure in epidemiological studies (1, 2). Adjustment for cumulative benzene exposure did not substantially change the results (relative risk, 47.4; 95% CI, 11.7–191.9 and relative risk, 61.3; 95% CI, 9.8–384.3, respectively).

Case-Control Study of BP. The 50 cases with BP were diagnosed, on average, 10 years (SD, 4.4) prior to 1992. Each of the 50 subjects with a past history of BP was considered in good health, with the exception of one person who had developed aplastic anemia 2 years after BP diagnosis and was transfusion dependent. Forty-one of 50 BP cases (82%) were no longer exposed to benzene, whereas the remaining cases were currently working in low-exposure areas of their factories (median, 0.5 ppm; range, 0.02–3.4 ppm as an 8-h TWA). Exclusion of the subject with aplastic anemia or of cases currently exposed to benzene from the analysis had minimal impact on the results.

Demographic characteristics among cases and controls were similar; cases were on average 46.3 years old (SD, 11.1), whereas

Table 1 *Benzene poisoning and subsequent risk of hematological malignancy and related disorders among 11,177 benzene-exposed workers in Shanghai, China*

BP	Person-yr[a]	No. of cases of all hematological disorders[b]	Relative risk[c] (95% CI)	No. of cases of ANLL/MDS	Relative risk[c] (95% CI)
No	122,620	7	1.0	3	1.0
Yes	848	3	42.3 (10.7–167.0)	2	70.6 (11.4–439.3)

[a] Follow-up from 1972–1987.
[b] ICD9 200–208 and MDS.
[c] Adjusted for age and sex.

controls were 44.7 years old (SD, 10.3). Forty-six % of the cases and controls were male, 32% of cases *versus* 36% of controls drank alcohol, and 36% of cases *versus* 40% of controls were current cigarette smokers.

No effect on BP risk was seen for the CYP2E1 *RsaI/PstI c2* allele (Table 2). There was, however, a 2.6-fold increased risk of BP for rapid *versus* slow $fe_{6\text{-OH}}$ and a 2.4-fold risk for subjects homozygous for the *NQO1* ^{609}C→T mutation compared with subjects who were heterozygous or wild type (Table 2). Risk estimates were minimally changed after each susceptibility factor was adjusted one for the other ($fe_{6\text{-OH}}$: OR, 3.0; 95% CI, 1.3–7.2 and *NQO1* ^{609}C-T: OR, 2.5; 95% CI, 1.0–6.5). Subjects with both a rapid $fe_{6\text{-OH}}$ and two copies of the *NQO1* ^{609}C→T mutation had a 7.6-fold increased risk of BP compared to subjects with a slow $fe_{6\text{-OH}}$ who carried one or two wild-type *NQO1* alleles (Table 3). Adjustment for body mass index, alcohol intake, and smoking status, none of which were significantly associated with BP risk, had only a minimal impact on the results (Tables 2 and 3).

There is some evidence that benzene metabolism is not linear as benzene exposure increases to relatively high levels (23). Hence, we determined whether these risk factors were similarly distributed among cases from factories with relatively high (*i.e.*, >10–25 or >25 ppm as an 8-h TWA) *versus* lower (*i.e.*, ≤10 ppm) average levels of benzene. A significantly larger proportion of high-exposure cases had a rapid $fe_{6\text{-OH}}$ [21 of 22 (95%)] compared to lower-exposure cases [13 of 26 (50%); $P = 0.001$, Fisher's exact test], but there was not a significant difference between these groups for the proportion of cases homozygous for the *NQO1* ^{609}C→T mutation [11 of 22 (50%) *versus* 8 of 26 (31%), respectively ($P = 0.24$)].

Discussion

In a retrospective cohort study of benzene-exposed workers in Shanghai, we found that a diagnosis of BP was strongly associated with subsequent development of hematological malignancies and nonmalignant related disorders, which is consistent with previous reports (24, 25). We then evaluated potential susceptibility factors for BP and found that subjects with a rapid $fe_{6\text{-OH}}$ who were homozygous for the *NQO1* ^{609}C→T mutation had a 7.6-fold risk of BP. This is the first report providing evidence that interindividual variation in metabolic processes among humans is associated with risk of a benzene-associated disease.

Table 2 *Impact of CYP2E1 genotype, $fe_{6\text{-OH}}$, and NQO1 genotype on risk for benzene poisoning in Shanghai, China, 1992*

Attribute	Cases,[a] no. (%)	Controls, no. (%)	OR[b] (95% CI)	OR_{adj}[c] (95% CI)
Total	50 (100)	50 (100)		
CYP2E1 RsaI/PstI				
c2c2	5 (10)	8 (17)	0.6 (0.2–2.1)	0.6 (0.2–1.9)
c1c1/c1c2	43 (90)	40 (83)	1.0	
$fe_{6\text{-OH}}$[d]				
Rapid	34 (71)	23 (48)	2.6 (1.1–6.0)	2.5 (1.1–6.0)
Slow	14 (29)	25 (52)	1.0	
NQO1 genotype[e]				
−−	20 (41)	11 (23)	2.4 (1.0–5.7)	2.6 (1.1–6.6)
++/+−	29 (59)	37 (77)	1.0	

[a] Data missing due to inability to amplify DNA or nondetectable 6-hydroxy chlorzoxazone metabolite levels.
[b] Adjusted for the matching variables age and sex.
[c] Adjusted for age, sex, body mass index, and alcohol and cigarette use. One additional control deleted due to missing data.
[d] Fractional excretion of chlorzoxazone as 6-hydroxy chlorzoxazone over 8 h.
[e] −−, homozygous for *NQO1* ^{609}C→T mutation: ++/+−, wild-type/heterozygous.

Table 3 *Joint effects of $fe_{6\text{-OH}}$ and NQO1 genotype on benzene poisoning risk in Shanghai, China, 1992*

$fe_{6\text{-OH}}$[a]	*NQO1* genotype[b]	OR[c] (95% CI) (No. cases)	OR_{adj}[d] (95% CI)
Slow	++/+−	1.0 (8)	1.0
Slow	−−	2.4 (0.6–9.7) (6)	2.7 (0.6–11.8)
Rapid	++/+−	2.9 (1.0–8.2) (21)	2.7 (0.9–8.0)
Rapid	−−	7.6 (1.8–31.2) (13)	7.8 (1.9–32.5)

[a] Fractional excretion of chlorzoxazone as 6-hydroxy chlorzoxazone over 8 h.
[b] −−, homozygous for *NQO1* ^{609}C→T mutation; ++/+−, wild-type/heterozygous.
[c] Adjusted for the matching variables age and sex.
[d] Adjusted for age, sex, body mass index, and alcohol and cigarette use. One additional control deleted due to missing data.

A control population of workers unexposed to benzene was used in the case-control study because benzene exposure theoretically could have increased chlorzoxazone metabolism through CYP2E1 induction (26). It is possible that use of an unexposed control group could have led to biased results if the proportion of subjects with a rapid $fe_{6\text{-OH}}$ and the *NQO1* ^{609}C→T mutation was not similar to that among healthy workers exposed to benzene. For example, workers with a slow $fe_{6\text{-OH}}$ or who were wild type/heterozygous for the *NQO1* gene might be more likely to leave the workplace if these characteristics were associated with chronic benzene-associated neurotoxic symptoms. This was examined in a parallel study of 44 benzene-exposed workers in Shanghai without a history of BP who were currently exposed to benzene at levels similar to those experienced by our BP cases (23). Fifty % of the currently exposed workers had a rapid $fe_{6\text{-OH}}$, and 18% were homozygous for the *NQO1* ^{609}C→T mutation, almost identical to the proportions observed in the unexposed control population used for this study (Table 2).

The *CYP2E1* DNA polymorphism was not associated with an increased risk of BP. Furthermore, among the workers currently exposed to benzene described above, this allele had no influence on levels of urinary hydroquinone, which is formed from phenol by hepatic CYP2E1 (Fig. 1), at any level of benzene exposure.[4] Although the minor *c2* allele has been associated with increased *in vitro* activity (17) and *in vivo* expression of mRNA in peripheral lymphocytes of subjects who drink ethanol (27), our data show no evidence that this allele significantly affects benzene metabolism or is associated with increased risk for BP.

This study used a urine-based assay to evaluate chlorzoxazone metabolism because a formal pharmacokinetic study with collection of multiple timed blood samples was not feasible. Three studies have

[4] Unpublished data.

reported a strong correlation between urine-based measures of 6-hydroxychlorzoxazone excretion and hepatic chlorzoxazone metabolism assessed by a full pharmacokinetic study (9, 28, 29), whereas one study did not (30). We were able to provide additional insight into the relevance of the $fe_{6\text{-}OH}$ for benzene metabolism among the group of workers currently exposed to benzene described above, finding that this measure was positively and significantly associated with urinary hydroquinone levels in workers exposed to >25 ppm benzene as an 8-h TWA.[4] This is consistent with our observation that almost all highly exposed BP cases had a rapid $fe_{6\text{-}OH}$. Nevertheless, these results cannot be attributed completely to hepatic CYP2E1 activity because chlorzoxazone hydroxylation, glucuronidation, and renal excretion as measured by the $fe_{6\text{-}OH}$ is likely to reflect additional factors (28, 30–32), some of which could play a role in benzene metabolism.

NQO1 has recently been characterized as an enzyme involved in the generation of antioxidant forms of ubiquinone (33) and α-tocopherol (34). In view of the antioxidant function of NQO1, the toxicological significance of the *NQO1* polymorphism may not be restricted to xenobiotics that generate reactive quinones, such as benzene, but may extend to a wide variety of agents that induce oxidative stress. The broader implications of the *NQO1* polymorphism for both chemoprotection and chemoprevention are worthy of future consideration.

In conclusion, a rapid $fe_{6\text{-}OH}$ and the *NQO1* $^{609}C\rightarrow T$ mutation were independently associated with risk of BP, a disorder that was associated with an exceptionally high risk of developing hematological malignancies and related disorders. Further study of susceptibility for hematotoxicity and hematological malignancy among workers with a history of occupational benzene exposure is warranted. In addition, the *NQO1* $^{609}C\rightarrow T$ mutation may be a risk factor for leukemia in general; this hypothesis is currently being explored (35).

Acknowledgments

We thank the personnel of the Department of Occupational Health, the Shanghai Hygiene and Anti-Epidemic Center, and the study subjects.

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